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Discovering Inner Ear and Central Auditory System Cellular Pathways That Might Contribute to Age-related Hearing Loss

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Abstract

Age-related hearing loss (ARHL) is a prevalent communication problem among senior citizens. Previous work (Tadros et al., 2008) has revealed genes which change their expression significantly with aging and hearing loss. However, this study is limited as it mainly focuses on apoptosis-related genes. Genes that regulate biological pathways other than apoptosis might also contribute to the development of age-related hearing loss, as suggested by some studies (Tadros et al., 2007; Souza et al., 2008). In order to circumvent this limitation and to better understand the underlying mechanisms of this communication problem, a free computational tool called Gene Set Enrichment Analysis (GSEA) is used to analyze the microarray data of aging cochlea and brain. Results show that most of the pathways which are up-regulated with aging/hearing loss in cochlea play a role in apoptosis and/or inflammation, suggesting that these two processes might be crucial for the development of ARHL. In contrast to the results from the cochlea, results for the aging brain indicate that cell cycle arrest is involved in deficits in the central auditory system with age.

Keyword: Age-related hearing loss, Gene Set Enrichment Analysis, Apoptosis, Cochlea, Auditory Midbrain, Microarrays

Abbreviations

APAF-1: apoptotic protease activating factor-1

ARHL: age-related hearing loss

AMI: acute myocardial infarction

ATM: ataxia telangiectasia mutated

Bcl-2: B-cell lymphoma 2

BAX: Bcl2-associated X protein

BLP: bacterial lipoprotein

DD: death domain

DISC: death inducing signal complex

ES: enrichment score

FDR: false discovery rate

FADD: Fas-associate death domain

GO: gene ontology

GSEA: gene set enrichment analysis

IL-1R: Interleukine-1 receptor

IRAK: IL-1R associated kinase

JNK: c-Jun N-terminal kinase

LPS: lipopolysaccharide

MSigDB: molecular signature database

NES: normalized enrichment score

PAMPs: pathogen-associated molecular patterns

PT: permeability transition

PUMA: p53 up-regulated modulator of apoptosis

RIP: receptor-interacting protein

RMA: robust multi-array average

ROS: reactive oxygen species

TIR domain: Toll/interleukin-1 receptor domain

TIRAP: TIR-domain-containing adapter protein

TNF: tumor necrosis factor

TRADD: TNF-receptor associated death domain

TRAFs: tumor necrosis factor receptor associated factors

TIMs: TRAF-interacting motifs

TLR: Toll-like receptor

Th cells: T helper cells

MyD88: myeloid differentiation factor 88

NF-kB: nuclear factor-kB

IkB: inhibitor of NF-kB

IKK: IkB kinase

NGF: nerve growth factor

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1. Introduction

1.1 Age-related Hearing Loss

In humans, age-related hearing loss (ARHL), or presbycusis, is a prevalent communication problem among senior citizens. It compromises the health of elderly persons and reduces the quality of their daily life, as it not only results in auditory deficiency, but also has psychological, physical and social consequences [1]. With the increased number of elderly people in our society, both in relative and absolute numbers, developing therapeutic treatments for ARHL becomes even more important, particularly in terms of reducing the load on our over-burdened health care system. As the therapeutic treatment of disease can be based on the understanding of the underlying cellular and molecular mechanisms behind it, numerous studies which focus on the pathological and physiological processes of ARHL have been conducted.

CBA/CaJ mice is often used as the model organism for the study of aging in the central auditory system. After correcting for the differences in the lifespans of mice and men, it shows a progressive hearing loss which is similar to presbycusis in humans (Frisina and Walton, 2006).

1.2 The Cochlea

The cochlea, whose structure consists of the inner and the outer hair cells, their supporting cells and the stria vascularis, is an important part of the auditory system in the mouse and the human inner ear [4]. Studies show that structural changes of the cochlea during the aging process lead to age-related hearing loss [1]. For example, it is often noted that ARHL is consistently associated with a decline in the number of outer hair cells and a smaller decrease in the numbers of inner hair cells [5]. Other lines of investigation point to the pathology of the stria vascularis, the specialized cochlear organ that produces endolymph, as a key component of ARHL (Schmiedt, 1996; Suryadevara et al.,2001). Thus, study of the underlying biological changes that occur with age in the cochlea is crucial to understanding the mechanisms of ARHL.

1.3 Apoptosis

1.3.1 Apoptosis and Age-related Hearing Loss

Study of ARHL is very difficult as it is an extremely complex system of processes involving the participation of multiple cellular pathways which are influenced by a

combination of different genetic and environmental factors [1]. It is highly speculated that apoptosis, the endogenous programmed cell death, plays an important role in ARHL.

1.3.2 Biological Significance of Apoptosis

Apoptosis, described first by Kerr, Wyllie, and Currie in 1972, is a distinct form of cell death which helps to maintain homeostasis in multi-cellular biological systems. It is of great biological significance: on the one hand, appropriate apoptosis is the requirement for the normal functioning of a wide variety of processes ranging from immune reactions, embryonic development to hormone-dependent atrophy [6,7]; on the other hand, dysfunction (or dysregulation) of apoptosis (either too little or too much) is associated with a variety of human diseases. For example, insufficient apoptosis may result in cancer, autoimmune diseases as well as spreading of viral infections. On the contrary, excessive apoptosis is thought to play a role in AIDS, ischaemic disease and neurodegenerative disorders like Parkinson's and Alzheimer's Disease [7,8].

1.3.3 Intracellular Morphological Features of Apoptosis

Cells undergo apoptosis following a series of controlled steps which eventually leads to cellular self-destruction in response to stimuli such as heat, radiation, hypoxia and

cytotoxic anticancer drugs [6,7]. The morphological changes of apoptosis include cell shrinkage (which is characterized by smaller cell size, dense cytoplasm and more tightly packed organelles), pyknosis (which results from chromatin condensation), karyorrhexis (fragmented nuclei), the plasma membrane blebbing and budding [4,6].

1.3.4 Apoptosis and Necrosis

Unlike necrosis, the unintentional form of cell death in which case inflammatory reactions are caused by the release of the cytoplasmic contents into the surrounding tissue as a result of disrupted cell membrane, the process of apoptosis is controlled in such a way that essentially, there is no inflammatory reaction associated with it nor does it cause damage to its surrounding cells [6,7]. This is largely due to the fact that apoptotic cells, which are quickly phagocytosed by macrophages or neighboring cells, do not release their cellular constituents into the adjacent tissues [6].

As different as necrosis and apoptosis are in their mechanisms, they are still closely connected to each other as they share morphological features and biological networks in common. Although both necrosis and apoptosis can be induced by similar stimuli, which one is going to be induced in cells depends largely on the degree and nature of the stimuli

[6]. Interestingly, there is even evidence indicating that an ongoing apoptotic process can be converted into a necrotic process under certain conditions [9,10].

1.3.5 Mechanisms of Apoptosis

Apoptosis is an extremely complex and sophisticated process. Generally, there are two types of apoptotic pathways: extrinsic (or death receptor) pathway and intrinsic (or mitochondrial) pathway. They converge at the final stage of apoptosis, named the execution phase, which starts with the activation of caspases [6].

1.3.5.1 Extrinsic Pathway

The extrinsic pathway is caused by the binding of specific ligands called pro-apoptotic ligands (such as Fas, TNF alpha and TRAIL) with death receptors that are located on the cell surface. Following ligand binding, receptors cluster and undergo the conformational changes. This induces a death domain and allows the recruitment of adaptor proteins such as Fas-associated death domain protein (FADD) or TNF-receptor associated death domain protein (TRADD) [8]. It then results in the formation of the protein complex, death inducing signal complex (DISC) and the recruitment of caspase 8. After caspase 8 is activated, the execution phase of apoptosis is initiated.

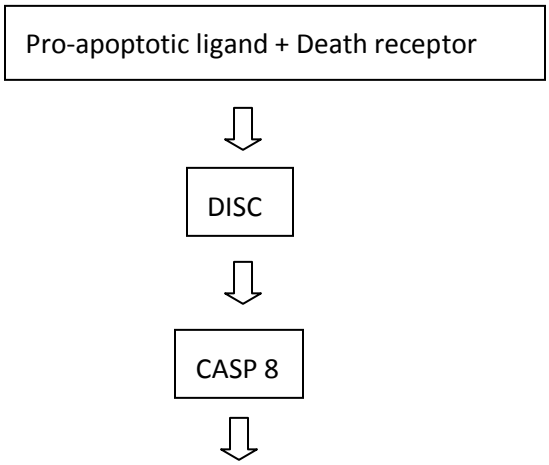

Figure 1. Extrinsic pathway of apoptosis

1.3.5.2 Intrinsic Pathway

The intrinsic pathway can be initiated by stimuli that act either negatively (withdrawal of cell survival signal, absence of certain growth factors) or positively (radiation, toxins, hypoxia, hyperthermia, viral infection, free radicals) inside the cells [6]. Upon stimulation, the mitochondrial inner transmembrane potential is reduced and the mitochondrial permeability transition (PT) pore is opened. This results in the release of pro-apoptotic proteins such as cytochrome c from the mitochondrial intermembrane space into the cytosol [6]. After that, cytochrome c binds the adaptor apoptotic protease activating factor-1 (Apaf-1) and procaspase-9, leading to the formation of a large multi-

protein complex called “apoptosome” which then causes the activation of caspase 9 and initiation of execution phase [6,8].

The intrinsic apoptosis pathway is regulated by the B-cell lymphoma 2 (Bcl-2) family of proteins which can be divided into two groups: pro-apoptotic and anti-apoptotic proteins. Pro-apoptotic proteins such as Bad, Bax and Bid tend to induce apoptosis while anti-apoptotic proteins such as Bcl-2, Bcl-X tend to resist to apoptosis [8]. Whether apoptosis will be activated or not is determined by the ratio of the two types of proteins [8].

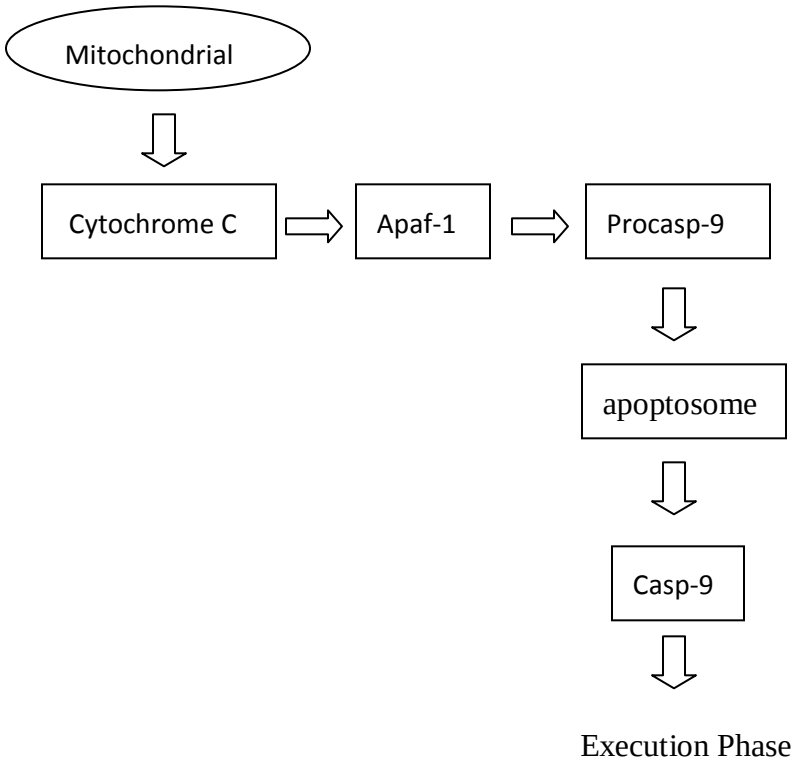

Figure 2. Intrinsic pathway of apoptosis

1.3.5.3 Execution Phase

Caspases, a family of proteins belonging to a group of enzymes known as cysteine proteases are the central executors and mediators of the execution phase of apoptosis [8]. There are two types of caspases: initiator caspases and effector (or executor) caspases which stay inactive within cells until being activated. Initiator caspases such as Caspase-2,8,9,10 which reside upstream in the pathway and are activated by the induction of either extrinsic pathways or intrinsic pathways or both, will in turn, activate downstream effector caspases such as Caspase-3,6,7. The activation of effector caspases, then leads to the cleavage of key cellular proteins such as cytoskeletal proteins, and this accounts for the morphological changes observed in cells undergoing apoptosis [8].

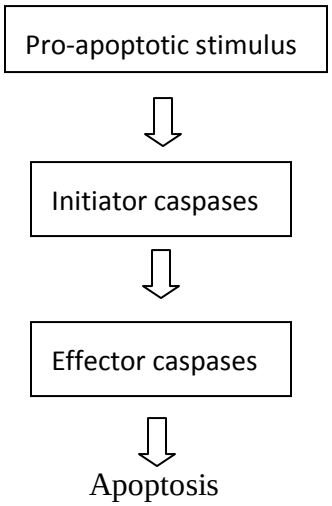

Figure 3. Execution phase of apoptosis

2. Data and Methods

2.1 Description of the Mouse Cochlea and Brain Microarray Data

Data used in this study are microarray mouse gene expression data from the cochlea and the inferior colliculus which are provided by Dr. Frisina's Lab. CBA/CaJ mice were used as the model organism for this study as this strain shows a progressive hearing loss that parallels that of presbycusis in humans, when accounting for the lifespan of each species (Frisina and Walton, 2006). Based on age and functional hearing measurements obtained from the auditory brainstem response recordings (ABR thresholds) and the otoacoustic emissions (DPOAE amplitudes), the whole sample set can be divided into four groups: young adult control with good hearing, middle-aged with good hearing, old with mild presbycusis and old with severe presbycusis [4]. One Affymetrix M430A high-density oligonucleotide array set containing 22,600 probe sets which analyze the expression of over 14,000 mouse genes was used for each sample [4]. Full experimental details can be found in Sherif F. Tadros, Mary D'Souza, Xiaoxia Zhu, Robert D. Frisina's paper: Apoptosis-related genes change their expression with age and hearing loss in the mouse cochlea, Apoptosis (2008) [4].

2.2 Data Normalization

The goal of high density oligonucleotide arrays is to detect differences of mRNA expression in response to genetic and environmental differences, which is also referred to as interesting variation [14]. However, non-biologically originated variations, also known as obscuring variations (or systematic variations) which are introduced during sample preparation, manufacture of the arrays, and the processing of the arrays (labeling, hybridization, and scanning) also exist and could mislead interpretation of the results [14]. Thus, before proceeding with a formal statistical analysis, obscuring variations should be reduced first by applying preprocessing steps such as data normalization. In this study, data is normalized by implementing the Robust Multi-array Average (RMA) method of Affymetrix Oligonucleotide Arrays R package, which is freely available (http://www.bioconductor.org). RMA is a normalization method which consists of three preprocessing steps: background correction, quantile normalization and a final summarization. It has been shown by other studies (Irizarry et al. 2003) that RMA outperformed AvDiff, the Li and Wong model-based expression index, dChip and MAS 5.0 in the following aspects: first of all, RMA shows higher precision without compromising its accuracy; second, estimations of fold change provided by RMA are

more consistent; third, results provided by RMA shows higher specificity and sensitivity, when using fold change analysis to detect differential expression patterns [12,14].

2.3 GSEA

2.3.1 Advantages Using GSEA

Gene Set Enrichment Analysis (GSEA) is a powerful analytical method for the interpretation of gene expression data and is incorporated into a software package that can be downloaded freely (http://www.broadinstitute.org/gsea). Traditional single gene analysis such as ANOVA, although useful, is limited by its low overlapping rate between different studies, its failure to capture significant genes with unifying biological themes as well as its deficiency to reveal underlying relationships between those genes. GSEA is designed to circumvent such limitations by analyzing gene expression data at the level of gene sets.

2.3.2 Overview

Based on gene sets which are defined based upon prior biological knowledge, such as published information about biochemical pathways or co-expression patterns in previous experiments and samples that fall into two classes, GSEA tries to find gene sets that are

associated with one of the two classes by determining whether the members of a gene set tend to occur toward the top (or bottom) of the class list [15].

2.3.3 Methods

There are three steps for the GSEA method. In the first step, an Enrichment Score (ES) is calculated to measure how well a given gene set is overrepresented towards the top or bottom of the entire ranked list. Then, the nominal P value which reflects the statistical significance of the ES is estimated using a phenotype-based permutation test. In the last step, ES is normalized to account for the size of each gene set, resulting in normalized enrichment score (NES) [16]. A false discovery rate (FDR) is also calculated with NES correspondingly.

2.3.4 Leading Edge Analysis

Even in gene sets with high ES, not all their members are up-regulated. Leading edge analysis is designed to find out the leading edge subset, a subset of genes that contribute most to the ES within a high scoring gene set [15]. Leading edge analysis might also help to reveal gene sets with the same biological function on the basis of common leading edge subsets that are shared by those high scoring gene sets.

2.3.5 Evaluation of Performances

In order to evaluate the performance of GSEA, several studies have been conducted (A. Subramanian et al., 2003). In the first study (Male vs. Female Lymphoblastoid Cells), gene sets on chromosome Y are found to be up-regulated in male samples as expected. Alternatively, X inactivation genes and female reproductive tissue expressed genes are found to be up-regulated in female samples. Results of two other studies analyzed by GSEA (p53 Status in Cancer Cell Lines and Acute Leukemias) are also biologically meaningful. In the last study, two lung cancer gene expression data sets obtained independently by different study groups (Boston group and Michigan group) are analyzed with GSEA as well as single gene analysis and their results are also compared. The study shows that similarities between results analyzed with GSEA of the two data sets are significantly higher than those analyzed with single gene analysis. Thus, it can be concluded that GSEA is superior to single gene analysis in both its reproducibility and interpretability [15].

2.3.6 Molecular Signature Database (MSigDB) and Biocarta

Since the performance of GSEA depends largely on how representative the given gene sets are to the actual biological processes, the definition and curation of such gene sets are of great significance [16]. Molecular Signature Database (MSigDB) which contains a collection of gene sets has been created on the GSEA website to assist its users. Gene sets can be generally divided into five categories: C1 positional gene sets which represent genes in the same chromosome or cytogenetic band; C2 curated gene sets which originate from publicly available databases of metabolic and signaling pathways such as KEGG, BioCarta and PubMed; C3 motif gene sets which are based on conserved regulatory motifs from comparative analysis of different species; C4 computational gene sets which is defined as sets of genes in expression neighborhoods of cancer-related genes; C5 GO gene sets which consist of genes annotated by the same GO terms (Subramanian, Tamayo, et al., 2005) [16]. The choice of specific gene sets should be based on data type and the goal of the study.

BioCarta, one of the C2 curated gene sets, is selected as the database for gene sets in this study. It seems to be the most appropriate choice for the present investigation, since it

maintains a good collection and classification of cellular pathways, including pathways that are involved in apoptosis, cell cycle regulation, metabolism and immunology.

3.Results

Since both old mice with mild presbycusis and old mice with severe presbycusis had a small sample size in the gene microarray data set of the present study, they are combined together as a single “old mice group” in order to have sufficient statistical power to carry out the study. Comparisons such as young mice versus old mice and middle-aged mice versus old mice are conducted using GSEA for both cochlea and brain gene microarray data. Leading edge analysis is then conducted for gene sets that are significantly up-regulated in old mice.

3.1 GSEA Result for Cochlear Data

The result of GSEA analysis for young mice versus old mice can be found in Table 1. Ten pathways are found to be significantly up-regulated in old mice (NOM p value < 0.05, FDR < 0.3): Toll-Like Receptor Pathway (TOLL), Signal transduction through IL1R (IL1R), TNFR1 Signaling Pathway (TNFR1), Signaling of Hepatocyte Growth Factor Receptor (MET), NF-kB Signaling Pathway (NF-kB), TNFR2 Signaling Pathway (TNFR2), TNF/Stress Related Signaling (Stress), FAS Signaling Pathway (CD95), Inhibition of Cellular Proliferation by Gleevec (GLEEVEC) and Induction of apoptosis through DR3 and DR4/5 Death Receptors (DEATH).

Symbol	NOM p-value	FDR q-value	Pathway Description
TOLL	0.002	0.040	Toll-Like Receptor Pathway
IL1R	0.002	0.034	Signal transduction through IL1R
TNFR1	0.023	0.147	TNFR1 Signaling Pathway
MET	0.008	0.240	Signaling of Hepatocyte Growth Factor Receptor
NFKB	0.022	0.238	NF-kB Signaling Pathway
TNFR2	0.010	0.225	TNFR2 Signaling Pathway
STRESS	0.029	0.258	TNF/Stress Related Signaling
FAS	0.024	0.282	FAS Signaling Pathway (CD95)
GLEEVEC	0.022	0.250	Inhibition of Cellular Proliferation by Gleevec
DEATH	0.049	0.284	Induction of apoptosis through DR3 and DR4/5 Death Receptors

Table 1. Gene sets that are up-regulated in old mice ($p < 0.05$, FDR < 0.3). Comparisons are conducted between young mice and old mice for the cochlear data.

From these results, it can be inferred that most of the pathways which are found to be up-regulated in old mice are closely connected with each other and fall into two major groups: Toll/IL-1R and TNF super-family related pathways.

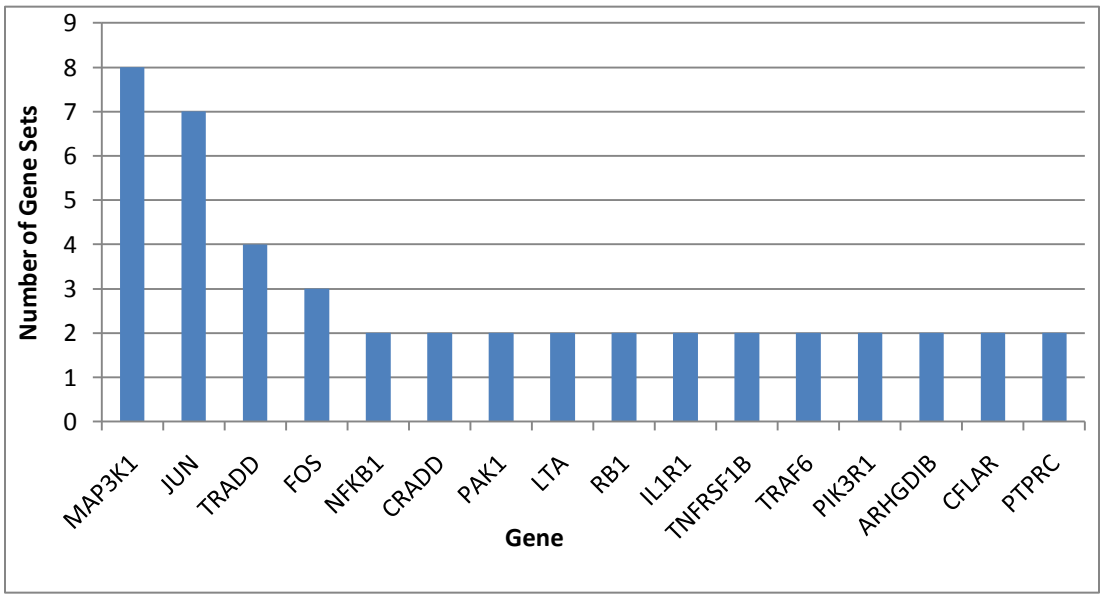

Figure 4. Leading edge analysis for the ten gene sets in Table 1.

Leading edge analysis is conducted for the ten gene sets in Table 1. Genes that appear at least twice are shown in Figure 4. Complete result for this analysis can be found in Appendix. Genes such as MAP3K1, JUN, CFLAR are well known apoptosis genes and showed significant gene expression differences in previous study (Tadros et al., 2008). TRADD and CRADD are also apoptosis-related. Although not found by previous study, they are statistically significant according to the result of this leading edge analysis. NFKB1, IL1R1 are genes that involved in inflammation and innate immunity.

Symbol	NOM p-value	FDR q-value	Pathway Description
INTRINSIC	0.000	0.012	Intrinsic Prothrombin Activation Pathway
IL6	0.004	0.103	IL 6 signaling pathway
TOLL	0.010	0.154	Toll-Like Receptor Pathway
NKT	0.024	0.166	Selective expression of chemokine receptors during T-cell polarization
IL2RB	0.008	0.145	IL-2 Receptor Beta Chain in T cell Activation
IL12	0.027	0.130	IL12 and Stat4 Dependent Signaling Pathway in Th1 Development
IL2	0.024	0.169	IL2 Signaling Pathway
NGF	0.030	0.276	Nerve growth factor pathway (NGF)
EPO	0.043	0.271	EPO Signaling Pathway
MET	0.031	0.246	Signaling of Hepatocyte Growth Factor Receptor

Table 2. Gene sets that are up-regulated in old mice ($p < 0.05$, FDR < 0.3). Comparisons are conducted between middle-aged mice with good hearing and old mice from cochlea data.

The result of GSEA analysis for middle-aged mice with good hearing and old mice can be found in Table 2. Ten pathways are found to be significantly up-regulated in old mice (NOM p value < 0.05, FDR < 0.3): Intrinsic Prothrombin Activation Pathway (INTRINSIC), IL 6 signaling pathway (IL6), Toll-Like Receptor Pathway (TOLL), Selective expression of chemokine receptors during T-cell polarization (NKT), IL-2 Receptor Beta Chain in T cell Activation (IL2RB), IL12 and Stat4 Dependent Signaling Pathway in Th1 Development (IL12), IL2 Signaling Pathway (IL2), Nerve growth factor pathway (NGF), EPO Signaling Pathway (EPO), Signaling of Hepatocyte Growth Factor Receptor (MET).

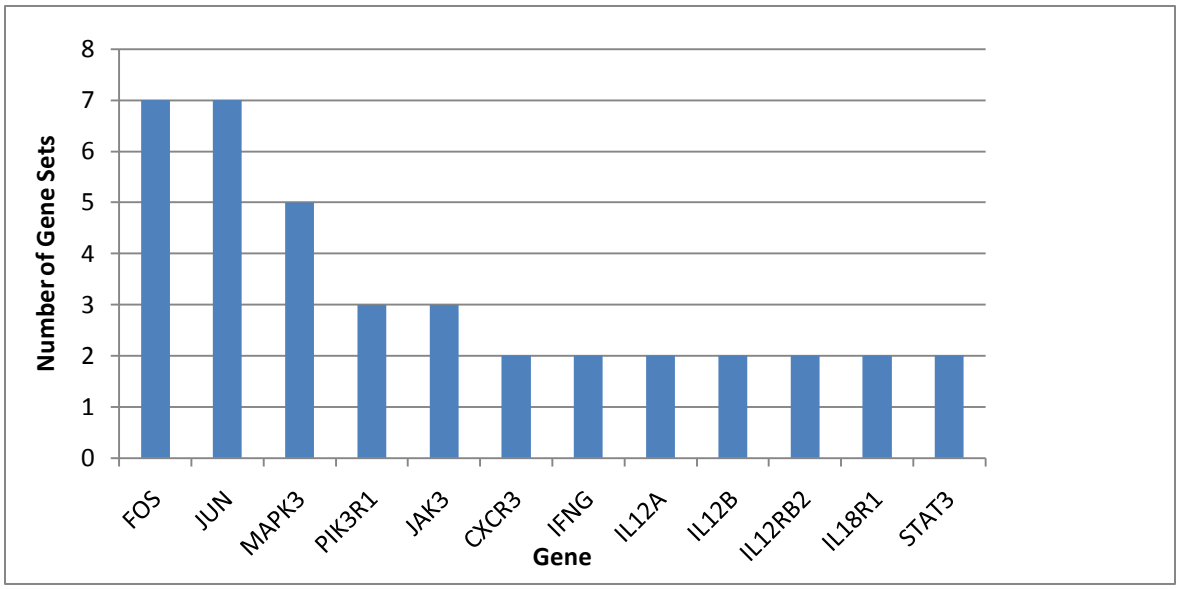

Figure 5. Leading edge analysis for the ten gene sets in Table 2.

Leading edge analysis is conducted for the ten gene sets in Table 2. Genes that appear at least twice are shown in Figure 5. Complete result for this analysis can be found in Appendix. Genes such as CXCR3, IL12A, IL12B, IL12RB2, IL18R1 and STAT3 are involved in T-cell differentiation and the development of adaptive immune system.

3.2 GSEA Result for Brain Data

Symbol	NOM p-value	FDR q-value	Pathway Description
P53	0.000	0.050	p53 Signaling Pathway
NKCELLS	0.004	0.042	Ras-Independent pathway in NK cell-mediated cytotoxicity
P38MAPK	0.004	0.032	p38 MAPK Signaling Pathway
AMI	0.004	0.043	Acute Myocardial Infarction
CSK	0.004	0.035	Activation of Csk by cAMP-dependent Protein Kinase Inhibits Signaling through the T Cell Receptor
ATM	0.020	0.136	ATM Signaling Pathway
NKT	0.030	0.140	Selective expression of chemokine receptors during T-cell polarization
G2	0.033	0.161	Cell Cycle: G2/M Checkpoint
G1	0.043	0.210	Cell Cycle: G1/S Check Point
IL1R	0.049	0.189	Signal transduction through IL1R
P53HYPOXIA	0.027	0.178	Hypoxia and p53 in the Cardiovascular system
RACCYCD	0.032	0.207	Influence of Ras and Rho proteins on G1 to S Transition

Table 3. Gene sets that are up-regulated in old mice for brain ($p < 0.05$, FDR < 0.3). Comparisons are conducted between middle-aged mice with good hearing and old mice for gene microarray data from the central auditory system.

The same comparisons are conducted for brain data. For the comparison young mice versus old mice, no individual gene set meets the threshold for statistical significance. The result of GSEA analysis for middle-aged mice with good hearing versus old mice can be found in Table 3. Twelve pathways are found to be significantly up-regulated in old mice (NOM p value < 0.05, FDR < 0.3): p53 Signaling Pathway (P53), Ras-Independent pathway in NK cell-mediated cytotoxicity (NKCELLS), p38 MAPK Signaling Pathway (P38MAPK), Acute Myocardial Infarction (AMI), Activation of Csk by cAMP-

dependent Protein Kinase Inhibits Signaling through the T Cell Receptor (CSK), ATM Signaling Pathway (ATM), Selective expression of chemokine receptors during T-cell polarization (NKT), Cell Cycle: G2/M Checkpoint (G2), Cell Cycle: G1/S Check Point (G1), Signal transduction through IL1R(IL1R), Hypoxia and p53 in the Cardiovascular system (P53HYPOXIA), Influence of Ras and Rho proteins on G1 to S Transition (RACCYCD).

P53, P53HYPOXIA, ATM and P38 MAPK are all closely associated with intrinsic apoptosis pathways. G1, G2 and RACCYCD pathways are responsible for cell proliferation.

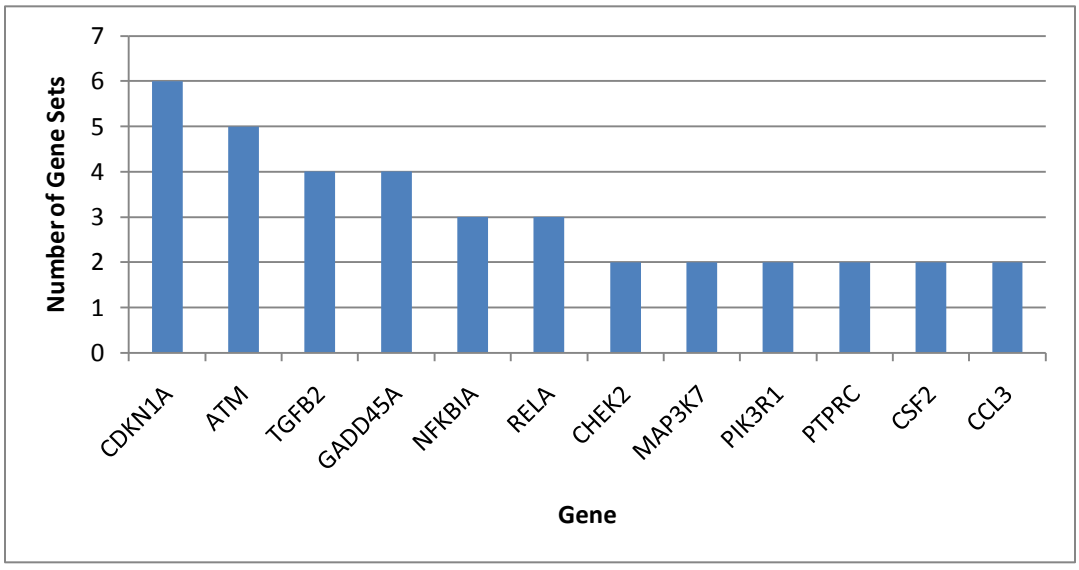

Figure 6. Leading edge analysis for the twelve gene sets in Table 3.

Leading edge analysis is conducted for the ten gene sets in Table 3. Genes that appear at least twice are shown in Figure 6. Complete result for this analysis can be found in Appendix. CDKN1A, ATM, TGFB2 and CHEK2 are involved in cell cycle arrest.

4. Discussion

4.1 Toll/IL-1R Pathway and Innate Immunity

Innate immunity is an evolutionarily conserved protective mechanism in multi-cellular organisms that provides the first line of defense for the host against invading pathogens. It is triggered upon recognition of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and bacterial lipoprotein (BLP) by pattern recognition receptors such as Toll-like receptor (TLR) and interleukin-1 receptor (IL-1R) [22]. Although previously being considered to be non-specific, innate immunity is later proved to have some specificity since it can not only detect infections, but is also capable of recognizing the type of invading pathogens [22]. Furthermore, it is a pre-requisite for the activation of acquired immunity [18].

Pathways that are pertinent to innate immunity include Toll-Like Receptor Pathway, signal transduction through IL1R and nuclear factor-kB (NF-kB) Signaling Pathway.

NF-kB plays a vital role in host defense mechanisms and it can be induced upon activation of Toll or IL-1R signaling pathways [20].

Although the extracellular domains of TLRs and IL-1Rs are significantly different, they have highly similar cytoplasmic domains, which is known as Toll/interleukin (IL)-1 receptor domain (TIR domain) [19]. Toll-Like Receptor Pathway and IL-1R Pathway are homologous transduction signaling pathways. Upon stimulation, Toll and IL-1R can induce the nuclear factor kB (NF-kB) pathway through MyD88/IRAK/TRAF6 pathway [20]. Myeloid differentiation factor 88 (MyD88) functions as the common mediator for Toll/IL-1R inducing NF-kB and apoptosis initiated by TLR2 [21,23]. MyD88 contains an N-terminal death domain and a C-terminal TIR domain [22]. The death domain interacts with the death domain of IL-1R-associated kinase (IRAK) and the TIR domain combines with the TIR domains of Toll and the IL-1R. IRAK is then activated, auto-phosphorylated [22]. After its disassociation from MyD88, it associates with another adaptor protein, called tumor necrosis factor (TNF) receptor-associated factors 6 (TRAF6) [22]. NF-kB pathways is activated after the phosphorylation of IkB protein by IKK kinases [22]. However, MyD88 is not indispensible for the activation of NF-kB since NF-kB can also be activated through a MyD88-independent pathway initiated by TLR3 and

TLR4, the mechanism of which is not fully understood [20]. It is shown that this MyD88 independent signaling is mediated by a novel adaptor protein called TIR-domain-containing adapter protein (TIRAP) (Janssens et al., 2002) [20]. MyD88 dependent and independent pathways converge at the formation of the IKK complex (Akira et al., 2001) [18].

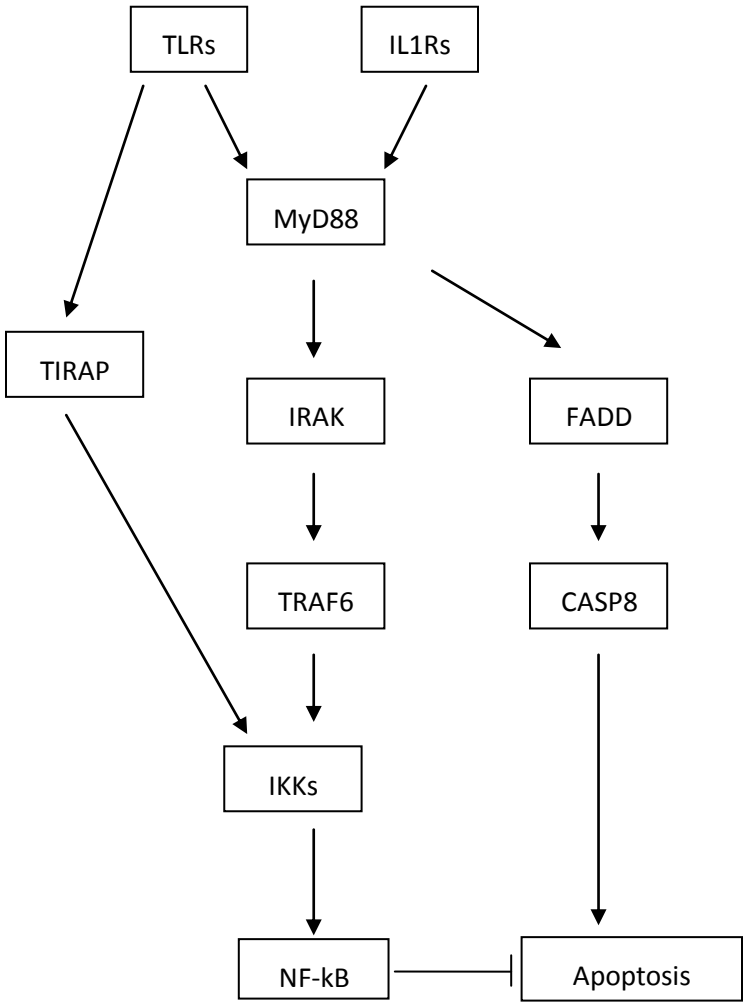

Figure 7. Toll/IL-1R signaling pathway leading to NF-kB and apoptosis initiated by TLR2. MyD88 works as the central mediator.

It is reported that the apoptotic signaling pathway can be activated by TLR2 via MyD88/FADD/Caspase 8 pathway (Aliprantis et al., 2000) [23]. FADD interacts with MyD88 through their death domains (DDs) [20]. FADD can then bind with pro-caspase 8, resulting in oligomerization, enzyme maturation and activation of apoptosis (Martin et al., 1998; Muzio et al., 1998b) [23]. Although involvement of IL-1 in apoptosis has been reported in diabetes mellitus (Sjoholm, 1998) and acute neurodegeneration (Rothwell et al.,1997), it is still not clear whether IL-1 mediates cell death through the MyD88/FADD/caspase 8 pathway [23]

4.2 NF-kB, Inflammation and Apoptosis

NF-kB signaling is the master regulator of inflammation. Before activated, NF-kB complexes bind to the inhibitory IkB proteins and stay in the cytoplasm [37]. Once stimulated, IkB proteins are phosphorylated and the NF-kB complex is translocated to the nucleus [37]. This leads to the activation of genes which are responsible for the induction of inflammation.

NF-kB signaling also can have anti-apoptotic actions in several ways [20,23]. First of all, inhibitors of apoptosis (IAPs) such as c-FLIP, Bcl-xL, c-IAP1, c-IAP2 and XIAP are activated by NF-kB signaling [37]. Furthermore, c-Jun N-terminal kinase (JNK), a well-

known kinase mediating apoptotic signals, can also be inhibited by NF-kB [37]. However, NF-kB has also been implicated to promote pro-apoptotic responses and induce cell death in some cell types (Baichwal and Baeuerle, 1997) [23].

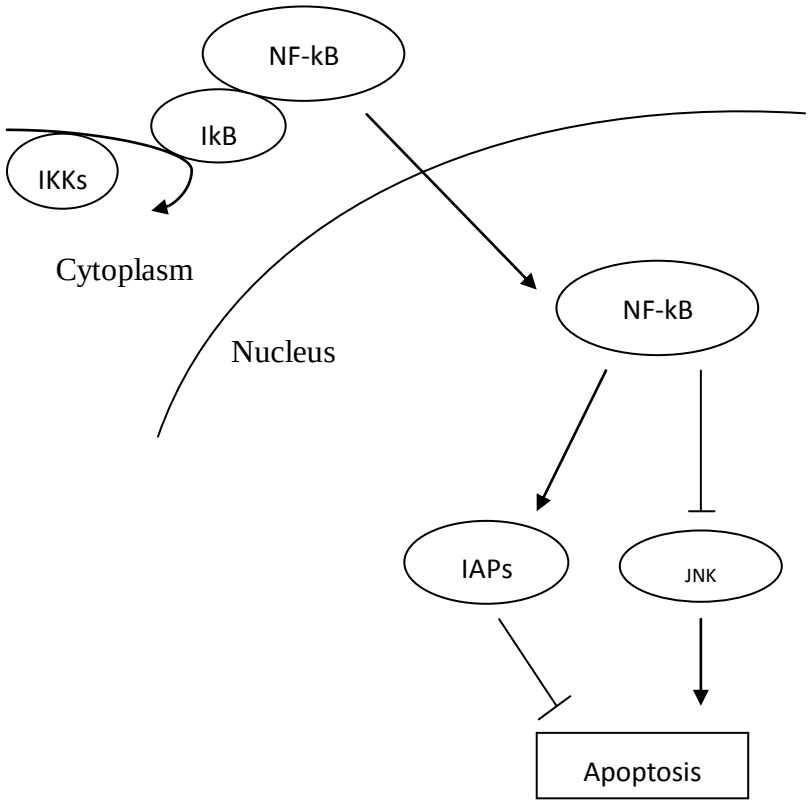

Figure 8. NF-kB inhibit apoptosis

4.3 TNF Superfamily, Induction of Apoptosis and NF-kB

Members of the TNF superfamily are known for their ability to regulate apoptosis, cell proliferation, survival and differentiation [24]. The existence of as many as 30 members of the TNF receptor superfamily has been revealed to date. Interestingly, it is shown that all members of the TNF superfamily activate NF-kB, which can suppress apoptosis.

TNFR1 Signaling Pathway (TNFR1), TNFR2 Signaling Pathway (TNFR2), TNF/Stress Related Signaling (Stress), FAS Signaling Pathway (CD95) and Death Pathway are pathways activated by members of the Tumor necrosis factor (TNF) super-family. The ligands, receptors and biological functions of these pathways can be found in Table 3.

Pathway	Ligand	Receptor	Induction of Apoptosis	Induction of NF-kB
TNFR1	TNF- α	TNFR1	Yes	Yes
TNFR2	TNF- β	TNFR2	Yes	Yes
TNF	TNF- α	TNFR1	Yes	Yes
Fas	FasL	Fas	Yes	Yes
Death	VEGI	DR3	Yes	Yes
Death	TRAIL	DR4, DR5	Yes	Yes

Table 3. Ligand, receptor and biological functions of TNF superfamily related pathways

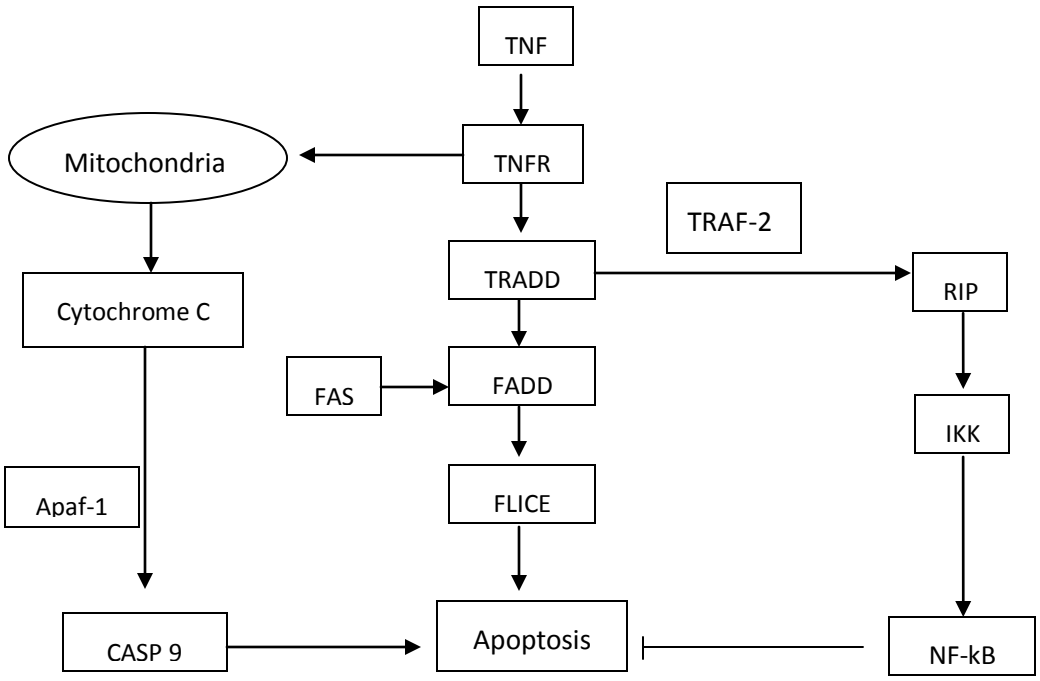

Figure 9. TNF signaling pathways leading to apoptosis and NF-kB

Note that these pathways function upstream for the activation of apoptosis, NF-kB, or both. The underlying mechanisms for the induction of apoptosis and NF-kB by the TNF superfamily related pathways will be discussed next and a graph that simplifies the whole process can be found in figure 9.

There are two types of TNF receptors: those that have a death domain (DD) in their cytoplasmic portion and those do not have a DD. TNFR1, Fas, DR3, DR4 and DR5 contain a DD and belong to the first type. They can lead to apoptosis by recruiting their DD with FADD and FLICE. TNFR1 induce apoptosis in slightly different way since its DD recruits TRADD first before the recruitment of FADD and FLICE. Apoptosis might

also be induced by the TNF family through mitochondrial dependent intrinsic pathways shown in Figure 9. TNFR2 does not have a DD and thus belongs to the second type. It has been shown that TNFR2 is still be able to mediate apoptosis through a DD independent way, the specific mechanism of which remains to be revealed (Ramesh et al.,2003; Haridas et al.,1998) [25,26].

NF-kB can be activated by all members of the TNF superfamily and this is mediated by a family of adaptors called tumor necrosis factor receptor associated factors (TRAFs) [27]. TNFRs associate with TRAFs through a conserved cytoplasmic motif termed TRAF-interacting motifs (TIMs). Receptor-interacting protein (RIP) is then recruited and this leads to the phosphorylation of IkB protein by IKK kinases and NF-kB activation.

4.4 T-cell Differentiation

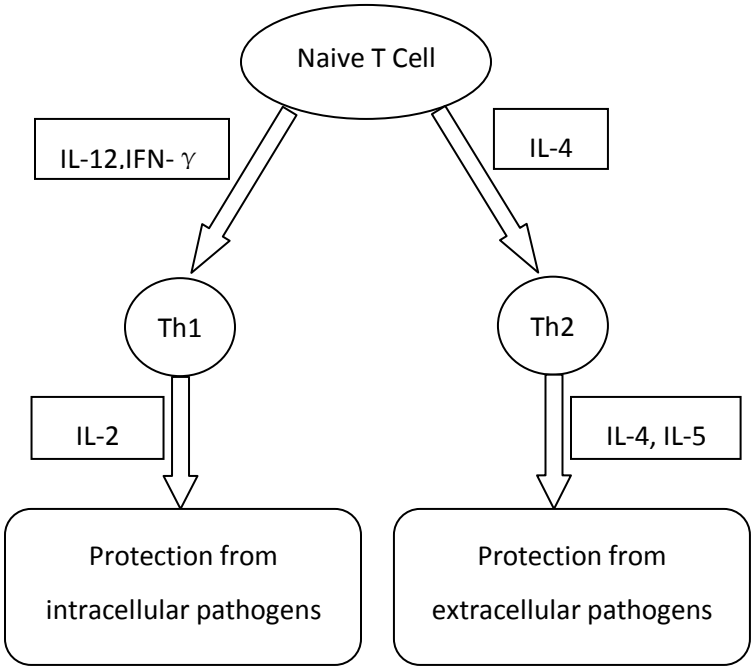

Figure 10. T-cell polarization

T cells, also known as lymphocytes, are a type of white blood cells that play an important role in adaptive immunity. Based on biological functions, T cells can be divided into several subsets such as T helper cells, cytotoxic T cells, memory T cells and natural killer T cells. T helper cells (Th cells) which assist in the immune response by secretion of small signaling protein molecules such as cytokines, can be differentiated into two major subgroups: T helper subset 1 cells (Th1) and T helper subset 2 cells (Th2).

Polarization of T helper cells depends largely on the type of cytokines during priming (See Figure 10). For instance, IL-12 and IFN- γ facilitate polarization of Th1 cells whereas IL-4 tend to promote differentiation of Th2 cells [36]. The two different types of cells are responsible for the protection against different pathogens. Th1 cells secrete IL-2 and provide protection for the host from intracellular pathogens. On the other hand, Th2 cells which secrete IL-4 and IL-5, are involved in the protection against extracellular pathogens [36].

Pathways such as IL12 and IL2 are up-regulated in old mice, indicating Th1 polarization is more likely than Th2 polarization in this case. Interestingly, for the analysis of young mice versus old mice, most of the pathways seem to be involved in innate immunity whereas for the analysis of middle-aged mice versus old mice, pathways are more connected to adaptive immunity. This is reasonable since innate immunity is activated before the adaptive immunity. Furthermore, activation of innate immunity is also required to activate adaptive immunity.

4.5 P53, Cell Cycle Arrest and Apoptosis

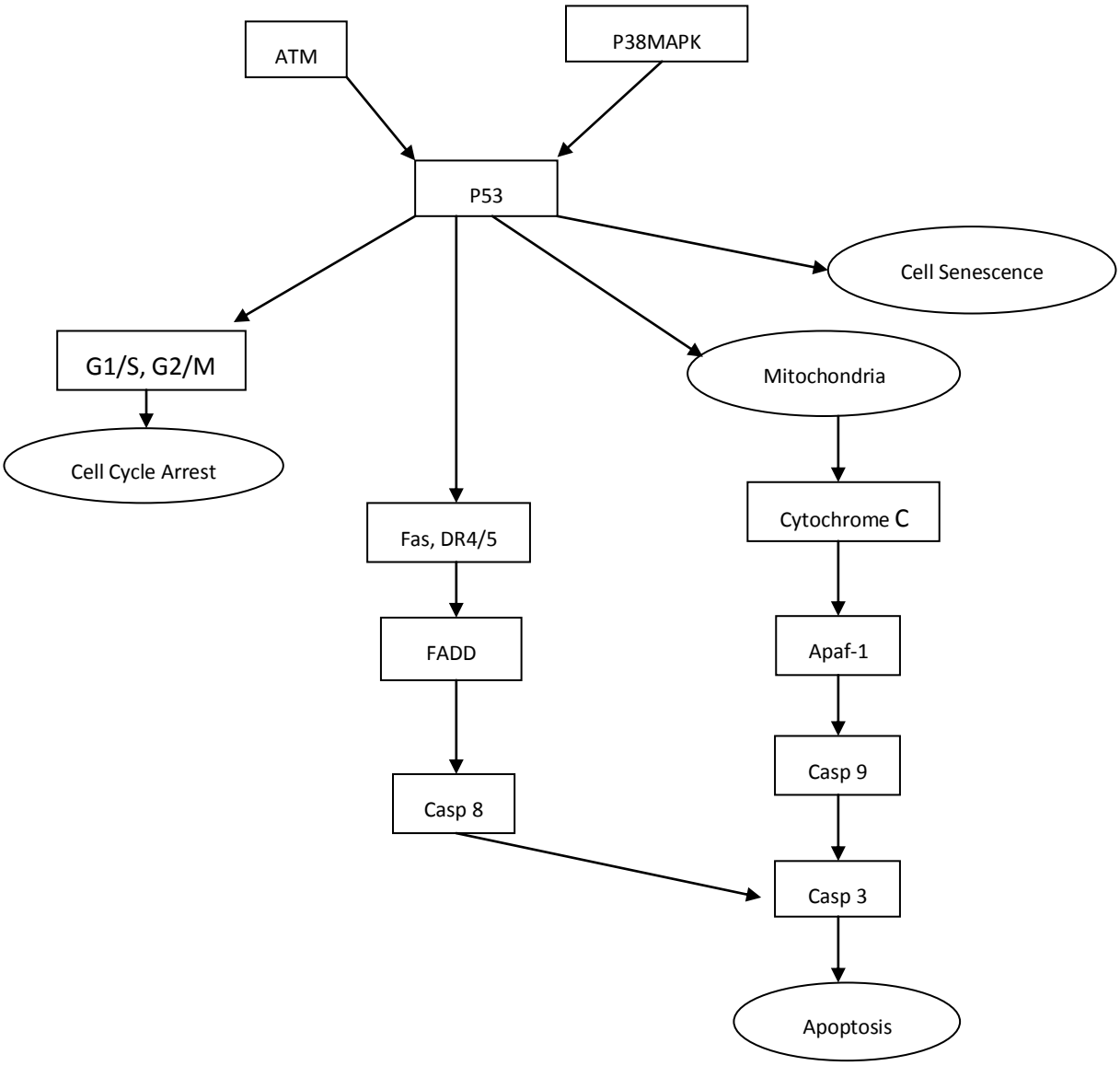

Figure 11. p53 pathway activate apoptosis, cell cycle arrest and cell senescence

The p53 signaling pathway can be activated in response to both intrinsic and extrinsic stresses such as DNA damage, hypoxia and oncogenic stimulation within cells [28,29]. Upon activation of the p53 pathway, cells have to choose to follow one of the three

pathways: cell cycle arrest, cell senescence or apoptosis [28]. Which one of the three programs is initiated within cells depends on a wide variety of factors, ranging from the nature of the stress signal, the types and location of the protein modifications on the p53 protein, to the pattern of gene expression in the specific tissue [28]. A graph that simplifies mediation of the p53 signaling pathway can be found in figure 11.

Ataxia telangiectasia mutated (ATM) has been reported to act upstream of p53 signaling pathways in an ionizing radiation initiated signal transduction study (Canman et al., 1998) [30]. In response to DNA damage, p53 is phosphorylated by ATM kinase, leading to the activation of downstream genes that regulate cell cycle checkpoints or apoptosis [31]. P38MAPK, another activator of p53, has been shown to be able to strengthen p53 activity, leading to cell cycle arrest or apoptosis (Takekawa et al, 2000) [32].

The p53 signaling pathways mediate apoptosis through both extrinsic and intrinsic pathways. Intrinsic apoptosis pathways are promoted when p53 interacts with mitochondria, up-regulating reactive oxygen species (ROS) and pro-apoptotic genes such as BCL2-associated X protein (BAX), p53 up-regulated modulator of apoptosis (PUMA) [33]. This results in cytochrome c release and activation of intrinsic apoptosis pathways. Extrinsic pathways of apoptosis are also mediated by p53. Death receptors such as

Fas/CD95 and DR5 are activated by p53 and this leads to the activation of extrinsic pathway of apoptosis [34].

Cells may arrest at cycle checkpoint to allow time for damage repair when exposed to genotoxic stress. In case that the damage is too severe to be repaired, the cell will undergo apoptosis. It is a protective mechanism that prevents further cellular damage. At least two checkpoints have been found: one at G1/S and the other at G2/M [35]. G1/S prevents damaged DNA from replication whereas G2/M is inhibited by damaged replicated DNA [35]. Evidence shows that p53 plays an important role in the G1 checkpoint since it is required for the activation of G1/S transition (Kastan, et al., 1991; Kuerbitz et al.,1992). P53 might be also involved in G2/M transition although it is not required.

4.6 Differences Between Brain and Cochlear Pathways

As discussed previously, one factor that might affect a cell's decision to undergo cell cycle arrest versus apoptosis is the cell type involved. Cells from different tissues might have different fates even in response to the same kind of stress signal. Thus, it is not surprising that pathways which are significantly up-regulated in old mice for brain tissue of the central auditory system are different from that for cochlear tissue samples. It seems

that up-regulated pathways in old mice for the cochlea such as TNFR1, TNFR2, STRESS, FAS and DEATH are well studied pathways leading to apoptosis. This might be evidence indicating cochlea cells undergo apoptosis involving cellular pathways common to other aging neural system. For brain, pathways such as G1, G2 and RACCYCD are significantly up-regulated in old mice. This might implicate the involvement of old brain cells in cell cycle arrest. However, it is not known whether this is due to tissue specificity or other, yet unidentified differences between the aging cochlea and aging auditory midbrain. Further experiments need to be conducted to sort out competing hypotheses.

5.Conclusions

GSEA is a powerful tool that allows us to analyze microarray data at the level of gene sets. This helps us to interpret results in a biologically meaningful way rather than struggling with a long list of genes that are up- or down-regulated with age or hearing loss, without unifying themes or biologically-relevant ways to categorize the data.

Results of the present study are in accordance with those of a previous study (Tadros et al., 2008), increasing the reliability of the current findings. For instance, genes that showed significant expression differences for the cochlea samples of all the subject groups such as members of TNF and TNF receptor family, Caspase family members and NF-kB related genes, are also important components of pathways which are implicated to be up-regulated in old mice, as previously discovered by Tadros et al. using different biostatistical data analysis approaches.

For the cochlea, most of the pathways identified in the present investigation seem to induce apoptosis and/or immune responses. Pathways regulating innate immunity seems to be significantly different for the analysis of young mice versus old mice, whereas pathways regulating adaptive immunity are significantly different for the analysis of

middle-aged mice versus old mice. This is in consistent with the fact that, across the lifespan, activation of innate immunity is required before the activation of adaptive immunity. Differences between results for the brain and cochlea may indicate tissue specificity concerning cell fate. It seems that cochlea cells are more likely to undergo apoptosis whereas brain cells tend to undergo cell cycle arrest; however, further experimentation needs to be conducted to verify this hypothesis.

6. Future Works

Pathways that induce apoptosis and NF-kB are both found to be significantly up-regulated with aging/hearing loss. Thus, it is quite possible that there is a balance between apoptosis and inflammation. How this balance is maintained might be crucial for the understanding of mechanisms of age-related hearing loss, for both the inner ear and central auditory system.

Since apoptosis pathways might play an important role in age-related hearing loss, inhibition of apoptosis might be a potential way to prevent or treat age-related hearing loss. However, this is limited by its harmful side effects since apoptosis is crucial for the maintenance of proper function of the organism, i.e., preventing oncogenesis. Thus, it is important to find the specific targets for age-related hearing loss that can lead to the discovery of effective therapies for this disease.

In the short term, future work could focus on the construction of genetic regulatory networks for apoptosis pathways using statistical methods such as Boolean Networks and Bayesian Networks. This might provide us deeper insights into interactions between

apoptosis related genes and their roles in the development and progression of age-related hearing loss.

Another research direction could be the comparison of regulation of pathways between brain and cochlea. It is expected that differences in regulation of pathways between brain and cochlea exist due to tissue specificity and cell type differences in these two levels of the mammalian auditory system. Results of such an investigation would reveal that cells in the cochlea and brain might have different fates. Cochlea cells are more likely to undergo apoptosis whereas brain cells, might choose to undergo cell cycle arrest, as suggested, by the present study. Understanding of this tissue specificity might help us to find biomarkers and potentially therapeutic agents, that when their pathways are blocked, they can specifically inhibit apoptosis in the cochlea without affecting other tissues.

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Appendix: Leading edge analysis

This appendix lists the complete result of leading edge analysis.

Gene	Number of Gene Sets
MAP3K1	8
JUN	7
CHUK	6
TRADD	4
FOS	3
NFKB1	2
CRADD	2
PAK1	2
LTA	2
RB1	2
MAP3K14	2
IL1R1	2
TNFRSF1B	2
TRAF6	2
PIK3R1	2
ARHGDIB	2
CFLAR	2
PTPRC	2
TOLLIP	1
TIRAP	1
TLR3	1
TGFB2	1
CASP6	1
CASP3	1
CASP7	1
CASP8	1
IL1RAP	1
IL1B	1
IL1A	1
MAP2K6	1
ACTA1	1
CYCS	1
MAPK8	1
TRAF1	1
BID	1
TRAF2	1
MAP3K7IP2	1

PXN	1
ATF1	1
BCL2	1
IL6	1
CEBPB	1
IL1RN	1
GAS2	1
BIRC4	1
BIRC3	1
TNFSF10	1
DUSP1	1
RAP1A	1
APAF1	1
IKBKB	1
CD14	1

Table 4. The result of leading edge analysis for the ten gene sets that are up-regulated in old mice for the GSEA analysis young mice versus old mice from cochlea samples.

Gene	Number of Gene Sets
FOS	7
JUN	7
MAPK3	5
PIK3R1	3
JAK3	3
CXCR3	2
IFNG	2
IL12A	2
IL12B	2
IL12RB2	2
IL18R1	2
STAT3	2
MAP3K7	1
MYC	1
IFNGR1	1
F11	1
IRAK1	1
F12	1
CD3G	1
F10	1
CD3D	1
ACTA1	1
SOCS3	1

LY96	1
RELA	1
F8	1
F9	1
SERPING1	1
CCR7	1
F5	1
CCR3	1
F2	1
LCK	1
CCR2	1
PROS1	1
CCL3	1
CCR1	1
BCL2L1	1
PXN	1
MAP3K7IP2	1
PTK2	1
FGA	1
PTK2B	1
KLKB1	1
BCL2	1
MAP3K1	1
SERPINC1	1
COL4A3	1
COL4A2	1
CEBPB	1
COL4A1	1
CBL	1
BAD	1
COL4A6	1
COL4A5	1
RAP1A	1
EPOR	1
CD14	1

Table 5. The result of leading edge analysis for the ten gene sets that are up-regulated in old mice for the GSEA analysis middle-aged mice versus old mice from cochlea samples.

Gene	Number of Gene Sets
CDKN1A	6
ATM	5
TGFB2	4
GADD45A	4
NFKBIA	3
RELA	3
CHEK2	2
MAP3K7	2
PIK3R1	2
PTPRC	2
CSF2	2
CCL3	1
MEF2A	1
KLRC3	1
IL18	1
CCR1	1
ELK1	1
ITGB1	1
CCL4	1
B2M	1
CDKN2D	1
IL4R	1
IL1RAP	1
IFNG	1
CD4	1
IL18R1	1
TGFBR1	1
STAT1	1
LAT	1
CCR5	1
CCR4	1
MAPK3	1
CCR2	1
KLRC1	1

Table 6. The result of leading edge analysis for the twelve gene sets that are up-regulated in old mice for the GSEA analysis young mice versus old mice from brain samples.